Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 143 (2007) 166–173
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Comparison of serological methods for the diagnosis of *Neospora caninum* infection in cattle

W. Wapenaar [a,*], H.W. Barkema [a], J.A. VanLeeuwen [a], J.T. McClure [a], R.M. O'Handley [b], O.C.H. Kwok [c], P. Thulliez [d], J.P. Dubey [c], M.C. Jenkins [c]

[a] *Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI, Canada C1A 4P3*
[b] *School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch, 6150 WA, Australia*
[c] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA*
[d] *Institut de Puériculture, 26 Boulevard Brune, F-75014 Paris, France*

Received 11 June 2006; received in revised form 10 July 2006; accepted 1 August 2006

Abstract

The aims of this study were to evaluate the performance and agreement of various commercial and in-house *Neospora caninum* antibody assays used in dairy cattle in North America, and to investigate reproducibility of two assays performed in different laboratories. From 1998 to 2005, three enzyme linked immunosorbent assays (ELISAs, a competitive ELISA-VMRD Inc., an indirect ELISA-Biovet Inc., and another indirect ELISA-Herdchek IDEXX Corp.), two indirect fluorescent antibody tests (IFATs, VMRD Inc., and in-house USDA) and one *N. caninum* agglutination test (NAT, in-house USDA) were utilized to test 397 randomly selected dairy cattle serum samples from 34 herds in eastern Canada for antibodies to *N. caninum.* The manufacturers' recommended cut-off values were used to evaluate test performance and agreement between tests. One IFAT (VMRD Inc.) performed well (sensitivity and specificity: 0.97 and 0.97, respectively) using reference sera ($n = 452$), therefore, results from this IFAT on the 397 samples could subsequently be used as the reference standard to calculate test characteristics for the other assays. Only 11% of the 397 sera were found to be *N. caninum*-positive with the IFAT. Prevalence-adjusted bias-adjusted kappa (PABAK) ranged from 0.06 to 0.99. Positive agreement was moderate to very good ($P_{pos} = 0.25$–0.96). Negative agreement was very good for all assays ($P_{neg} > 0.94$) except NAT ($P_{neg} = 0.66$). Sensitivity was ≥ 0.89 for all assays except the NAT, which had a significantly lower sensitivity (0.66). Specificity was high (>0.94) for all assays except for one indirect ELISA (specificity = 0.52). This indirect ELISA did not perform satisfactorily when used in 1998, but an improved version of the ELISA performed as one of the best assays in 2004. Reproducibility of the competitive ELISA was excellent, but the reproducibility of the indirect ELISA that was improved was low (concordance correlation coefficient = 0.90 and 0.36, respectively). The performance characteristics observed for most assays in this study make them useful for screening antibodies to *N. caninum* in cattle.

Keywords: *Neospora caninum*; ELISA; Indirect fluorescent antibody test; Agglutination test; Gold standard; Test comparison

1. Introduction

Definitive diagnosis of *Neospora*-associated abortions is based on examination of the aborted foetus, including observation of characteristic lesions, combined with immunoperoxidase staining or PCR in foetal tissues

* Corresponding author. Tel.: +1 902 566 0437; fax: +1 902 566 0823.
E-mail address: wwapenaar@gmail.com (W. Wapenaar).

0304-4017/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2006.08.007

(Wouda et al., 1997). However, in many instances, foetal material is not available. In these situations, a presumptive diagnosis can be achieved based on *Neospora caninum* antibody detection by serological assays.

Serological techniques available to detect specific antibodies to *N. caninum* infection include indirect fluorescent antibody tests (IFATs), a variety of enzyme linked immunosorbent assays (ELISAs), and a *N. caninum*-agglutination test (NAT). In addition to the use of these assays for abortion-related diagnosis, the different serological techniques are also widely used in epidemiological studies and herd-level control efforts regarding *N. caninum*.

While several commercial tests are available, many laboratories with a *N. caninum* research program, or that are involved with *N. caninum* diagnostics, often develop their own immuno-assays, using their own cut-off values or other criteria for interpretation. Thus, the interpretation of data from different laboratories depends on the quality of serological diagnostic tools and the utilized cut-off value, making it difficult to compare data from different laboratories using different (or even the same) techniques. In 1997, substantial variability between test results was observed in an evaluation of five ELISAs and one IFAT used in the US (Dubey et al., 1997). However, the sample size used to evaluate these tests was small (33 *N. caninum*-positive and 37 *N. caninum*-negative sera). In contrast, a large study recently showed a high level of agreement in the interpretation of test results from six commercial and six in-house tests used in Europe (Von Blumröder et al., 2004). A Canadian study using field samples from beef cattle found good agreement between two ELISAs ($\kappa = 0.76$) but much lower agreement ($\kappa = 0.46$) between the same two ELISAs and an agglutination test (Waldner et al., 2004). However, in this study there was no information about the 'true' status of the sera.

The aims of this study were (1) to evaluate the performance and agreement of various commercial and in-house *N. caninum* antibody assays used in dairy cattle in North America, and (2) to investigate reproducibility of two assays performed in different laboratories.

2. Materials and methods

2.1. Sample population

Serum samples were randomly collected from lactating dairy cows in three Canadian provinces (New Brunswick, Nova Scotia, and Prince Edward Island) as part of a large study in Canada in 1998 (Keefe and VanLeeuwen, 2000). No abortion history of these animals was known. From a sample pool of 2594 samples from 90 herds, 183 seronegative and 214 seropositive samples, as determined by a single serological assay ELISA-B1 (*N. caninum* indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada), were randomly selected. The 397 sera in the final sample population originated from 34 herds. After collection in 1998, samples were stored in a −20 °C freezer in sealed containers to prevent dehydration.

2.2. Laboratory analyses

In total, three ELISAs(A, B and C), two IFATs(A and B) and one NAT were utilized to test the sample population for antibodies to *N. caninum* (Table 1). The

Table 1
Summary information and identification of *Neospora caninum* antibody assays used in North America that were evaluated in the study

Test ID	Trademark[a]	Method	Diagnostic laboratory[a]	Year of analysis	Antigen
Commercial					
IFAT-A	VMRD Inc. (Baszler et al., 1996)	IFAT	VMRD	2005	Whole tachyzoites
ELISA-A1	VMRD Inc.	Competitive ELISA	AVC	2003	Surface protein antigen captured
ELISA-A2	(Baszler et al., 2001)	(VMRD, 2006)	VMRD	2005	using a monoclonal antibody
ELISA-B1	Biovet Inc.	Indirect ELISA	Biovet	1998	Sonicated lysate of tachyzoites
ELISA-B2	(Pare et al., 1995)	(Biovet, 2006)	AVC	2004	
ELISA-C	HerdChek IDEXX (Pare et al., 1995)	Indirect ELISA	AVC	2004	Sonicated lysate of tachyzoites
In-house					
IFAT-B	(Dubey et al., 1988)	IFAT	USDA	2004	Whole tachyzoites
NAT	(Romand et al., 1998)	NAT	USDA	2004	Whole tachyzoites

[a] VMRD Inc., Pullman, WA, USA; Biovet Inc., St. Hyacinthe, Que., Canada; IDEXX Corp., Westbrook, ME, USA; USDA, Beltsville, MD, USA; AVC, Atlantic Veterinary College, Charlottetown, Prince Edward Island, Canada.

assays were performed according to the manufacturers' instructions or, in the case of in-house tests, according to respective laboratory protocols. ELISA-B2 had two recommended cut-off values, giving results classified as 'suspicious' if the sample-to-positive (S/P) ratio was between these two cut-off values.

In order to determine the reproducibility of two assays in different laboratories, the competitive ELISA (ELISA-A1 and ELISA-A2) and the indirect ELISA (ELISA-B1 and ELISA-B2) were performed twice. The first competitive ELISA (ELISA-A1) was performed at the Atlantic Veterinary College (AVC, Prince Edward Island, Canada) in 2003, and ELISA-A2 by the VMRD Laboratory (Pullman, WA, USA) in 2005. The first indirect ELISA (ELISA-B1) was performed in 1998 by the Biovet Laboratory (Quebec, Canada), and ELISA-B2 by AVC in 2005. These two assays were selected for this reproducibility assessment because they are two commonly used commercial ELISAs for *N. caninum* in North America.

2.3. Reference standard determination

Determination of test operating characteristics of the assays under evaluation in this study required a comparison of the test results with the 'true state' of the sample population. In order to determine this 'true state' in the current study, one of the test assays was validated on 452 reference sera provided by the *Neospora*-research group in Europe (Von Blumröder et al., 2004). As the volume of serum from samples in this reference panel was limited, only one test, the IFAT-A (Table 1), could be performed with these reference sera. If IFAT-A resulted in a high sensitivity and specificity using these reference sera, then the results from IFAT-A on the sample population could be used as reference standard for the sample population. These European reference sera were considered to be a 'reference panel' because the sera underwent a comparison of 12 European serological methods, reported by Von Blumröder et al. (2004). A 'true state' of nature of the 452 sera (286 *N. caninum*-negative and 166 *N. caninum*-positive) was determined by 'majority of test-outcome', based on the decision (positive or negative) of the majority of the 12 assays. From these 452 samples, 234 samples (142 *N. caninum*-negative and 92 *N. caninum*-positive) also had 'pretest' information on the 'true state' of nature of the sample, based on historical information from the animal (e.g. the animal was known to have aborted a foetus infected with *N. caninum*). Therefore, the results of IFAT-A could be validated with the 'true state' of the whole panel of reference sera, and the 234 samples with additional information.

For interpreting the results of the IFAT-A on the 452 reference sera (and 397 sample sera), a 1:200 serum dilution was used, and the intensity of fluorescent antibody bound to the periphery of the tachyzoite was recorded on a categorical scale from negative to strong fluorescence (0–4+) by an experienced reader (Dubey et al., 1996). Fluorescence of the periphery of the tachyzoite recorded as 0 or 1+ was considered as *N. caninum*-negative result. Fluorescence of the periphery of the tachyzoite recorded as >1+ was considered *N. caninum*-positive. A 'strong positive' response was a strong fluorescence of the periphery of the tachyzoite (>3+), and a moderate fluorescence of the periphery of the tachyzoite (≤3+) was interpreted as a 'weak positive' response. Based on results of IFAT-A on these reference sera, the IFAT-A was subsequently considered as reference standard for assessing the operating characteristics of the five other assays on the sample population used in our study.

2.4. Data analysis

Because the more frequently used Cohen's kappa becomes unstable when prevalence of disease or infection is very low (<0.2) or high (>0.8) (Dohoo et al., 2003), prevalence-adjusted bias-adjusted kappa (PABAK) (Byrt et al., 1993) was used to assess test agreement between eight assay results (six assays, with two assays conducted at two laboratories). Positive and negative agreements were calculated to assess in more detail where disagreements among test results occurred (Cicchetti and Feinstein, 1990). Based on the positive–negative classification of the reference standard (IFAT-A) for the sample population, sensitivity (Se), specificity (Sp) and Youden's index (Youden, 1950) of the other five assays were calculated. For the ELISA results, two-graph receiver operating characteristic (TG-ROC) plots were used to assess each assay's ability to discriminate the sample population in relation to its cut-off value (Greiner et al., 2000).

To assess reproducibility between ELISA-A1 and ELISA-A2, and between ELISA-B1 and ELISA-B2, Lin's concordance correlation coefficient (CCC) (Lin, 1989) was calculated and a concordance correlation plot was utilized to examine absolute agreement. Stata version 8 (Stata Corporation, College Station, TX, USA) was used for the statistical analyses of the data.

Table 2
Test characteristics and Youden's index (J) of five *Neospora caninum* antibody assays using recommended cut-off values on sera from 397 dairy cows

Test[a]	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	*J* (95% CI)
ELISA-A1	≥30%	0.89 (0.79–0.98)	0.98 (0.97–1.00)	0.87 (0.77–0.96)
ELISA-A2	≥30%	0.89 (0.79–0.98)	0.99 (0.97–1.00)	0.87 (0.78–0.97)
ELISA-B1	≥0.60	1.00 (1.00–1.00)	0.52 (0.47–0.57)	0.52 (0.47–0.57)
ELISA-B2[b]	≥0.45	0.91 (0.82–0.99)	0.98 (0.96–0.99)	0.89 (0.80–0.97)
ELISA-B2[b]	≥0.60	0.89 (0.79–0.98)	0.99 (0.99–1.00)	0.88 (0.79–0.97)
ELISA-C	≥0.50	0.93 (0.86–1.00)	0.94 (0.91–0.96)	0.87 (0.79–0.95)
IFAT-B	1:100	0.93 (0.86–1.00)	0.96 (0.94–0.98)	0.89 (0.81–0.97)
NAT	1:25	0.66 (0.52–0.80)	0.99 (0.97–1.00)	0.64 (0.50–0.78)

[a] ELISA-A1 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-B2 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-C = indirect ELISA, IDEXX Corp., Westbrook, ME, USA; IFAT-B = IFAT, USDA, Beltsville, MD, USA; NAT = *N. caninum* agglutination test, USDA, Beltsville, MD, USA.

[b] Inconclusive cut-off value, a 'suspicious' range is recommended to be used in between cut-off values 0.45 and 0.60, and for this reason, test characteristics for both cut-off values were established.

3. Results

3.1. Test performance of IFAT-A relative to European reference panel

For reference sera with 'pretest' information ($n = 234$), Se and Sp of the IFAT-A were 0.99 (95% CI: 0.97–1.00) and 0.97 (95% CI: 0.95–1.00), respectively. When using the 'majority of test-outcome' information ($n = 452$), the Se and Sp were 0.97 (95% CI: 0.94–1.00) and 0.97 (95% CI: 0.95–0.99), respectively. The IFAT-A response was more frequently 'strong positive' on samples of experimentally infected cows (88%), compared to cows that experienced a natural infection (32%) ($P < 0.01$).

3.2. Test performance of five assays relative to IFAT-A as the reference standard

Only 11% ($n = 44$) of the 397 sera in the sample population were found to be *N. caninum*-positive with IFAT-A. Using IFAT-A results as the reference standard for the sample population, Se and Sp ranged from 0.66 to 1.00 and 0.52 to 0.99, respectively (Table 2). The highest Youden's index was 0.89 for both ELISA-B2 and IFAT-B.

In Fig. 1, TG-ROC graphs illustrate the change in Se and Sp in relation to the S/P ratio (ELISA-B1, ELISA-B2 and ELISA-C) or inhibition percentage (ELISA-A1and ELISA-A2). A cut-off value that realizes equal test parameters (Se = Sp) can be obtained at the intersection point of the two curves in each graph. The cut-off value recommended by the manufacturer of ELISA-C was close to the intersection point, at a Se and Sp of 0.93 and 0.94, respectively (Fig. 1).

The PABAK indicated the least agreement (0.06) between ELISA-B1 and NAT, and maximum agreement (0.97–0.98) was observed amongst ELISA-A1, ELISA-A2 and ELISA-B2 (Table 3). Negative agreement was 'substantial' to 'high' between all tests (0.66–0.99), according to Cicchetti and Feinstein (1990). Positive agreement ranged from 0.25 (between ELISA-B1 and NAT) to 0.96 (between ELISA-A1 and ELISA-A2).

3.3. Reproducibility

Almost perfect concordance was observed between ELISA-A1 and ELISA-A2 (Fig. 2), with the line of best fit only slightly deviating from the line of perfect concordance, which is in agreement with the high CCC (0.90, 95% CI 0.89–0.92). The line of perfect concordance between ELISA-B1 and ELISA-B2 (Fig. 3) was dissimilar to the best line of fit, which is in agreement with the observed CCC (0.36, 95% CI 0.30–0.41).

4. Discussion

The results of this study show good performance characteristics of IFAT-A compared to the European reference panel and demonstrate agreement between several serological assays used in North America. The decision as to which assays to include in this study was determined by their relative frequency of use in research and diagnostic laboratories in North America. The number of samples used was sufficient to give a good estimate of test performance, although a higher number of positive samples would have improved the precision of sensitivity results.

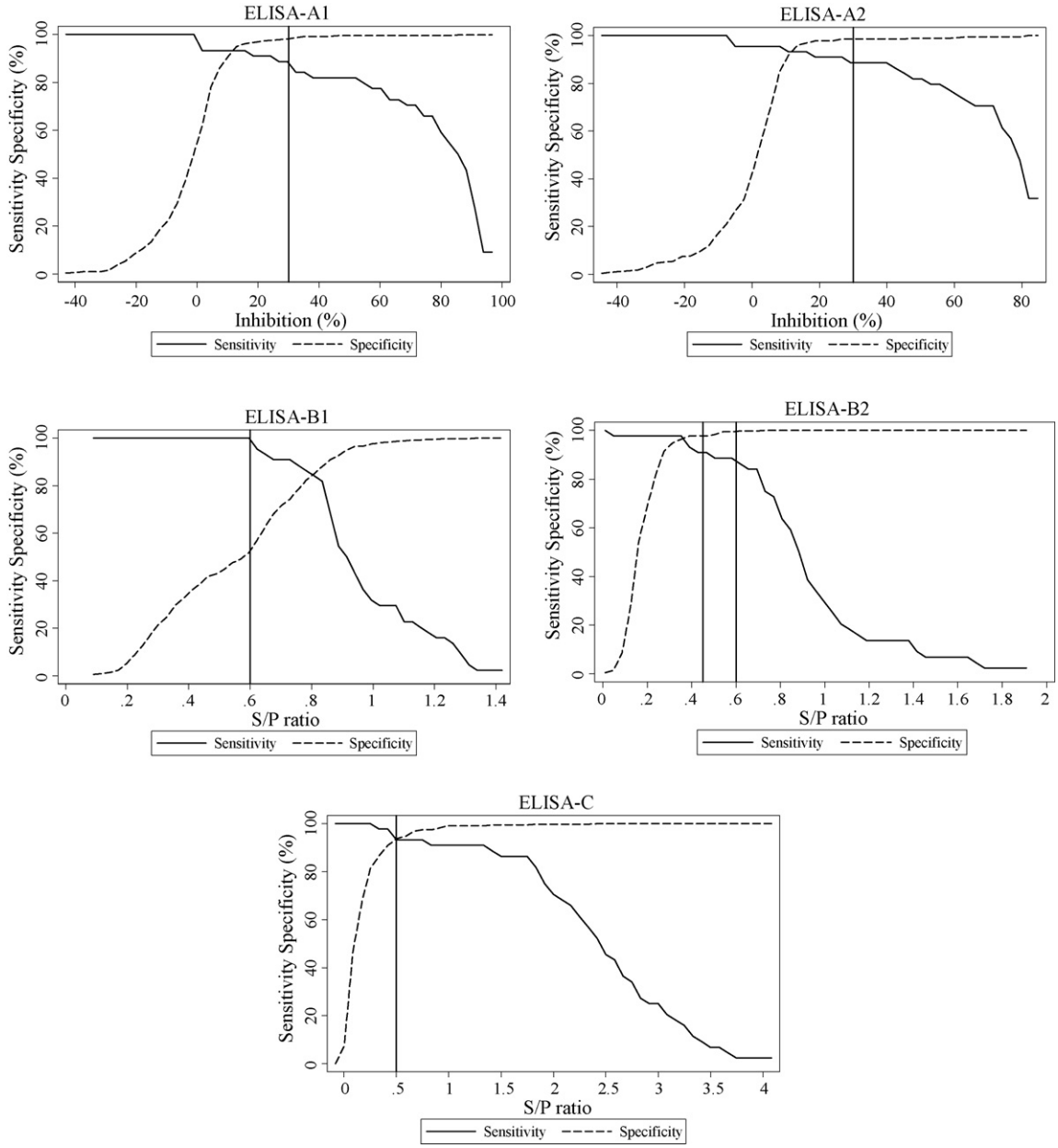

Fig. 1. Two-graph receiver operating characteristic (TG-ROC) plots for five *Neospora caninum* ELISAs (ELISA-A1, ELISA-A2, ELISA-B1, ELISA-B2, ELISA-C) compared to IFAT-A, with vertical line indicating cut-off value recommended by the manufacturer, on sera from 397 dairy cows. IFAT-A = IFAT, VMRD Inc., Pullman, WA, USA; ELISA-A1 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-B2 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-C = indirect ELISA, IDEXX Corp., Westbrook, ME, USA.

IFAT-A had a high Se and Sp in both 'pretest' and 'majority' reference samples, confirming that it was possible to use IFAT-A as reference standard to estimate Se and Sp for the other assays. The experimentally infected animals had high IFAT responses, while the naturally infected animals had a significantly lower IFAT response, which is in agreement with a study by Matsushita et al. (1987). This emphasizes the importance of validating assays with field samples, as using only experimental samples for validation may overestimate test performance.

Test performance of most assays was good, except for ELISA-B1 and NAT. The Se and Sp of a particular test can be manipulated by choosing different cut-off

Table 3
Prevalence-adjusted, bias-adjusted kappa (PABAK), positive (P_{pos}) and negative (P_{neg}) test agreement values[a], with bold printed numbers indicating almost perfect agreement, on sera from 397 dairy cows

Test[b]		ELISA-A1	ELISA-A2	ELISA-B1	ELISA-B2[c]	ELISA-C	IFAT-B	NAT
IFAT-A	PABAK	**0.94**	**0.95**	0.14	**0.96**		**0.91**	**0.90**
	P_{pos}	**0.87**	**0.89**	0.34	**0.92**	0.76	**0.83**	0.74
	P_{neg}	**0.98**	**0.99**	0.68	**0.99**	**0.96**	**0.98**	**0.97**
ELISA-A1	PABAK	–	**0.98**	0.15	**0.97**	**0.88**	**0.88**	**0.88**
	P_{pos}		**0.96**	0.35	**0.92**	0.78	0.77	0.70
	P_{neg}		**0.99**	0.69	**0.99**	**0.97**	**0.97**	**0.97**
ELISA-A2	PABAK		–	0.18	**0.98**	**0.88**	**0.89**	**0.88**
	P_{pos}			0.34	**0.94**	0.78	0.79	0.69
	P_{neg}			0.68	**0.99**	**0.97**	**0.97**	**0.97**
ELISA-B1	PABAK			–	0.13	0.23	0.18	0.06
	P_{pos}				0.32	0.45	0.39	0.25
	P_{neg}				0.68	0.71	0.69	0.66
ELISA-B2	PABAK				–	**0.88**	0.91	**0.90**
	P_{pos}					0.78	**0.81**	0.75
	P_{neg}					**0.97**	**0.97**	**0.97**
ELISA-C	PABAK					–	**0.84**	**0.80**
	P_{pos}						0.74	0.59
	P_{neg}						**0.95**	**0.94**
IFAT-B	PABAK						–	**0.83**
	P_{pos}							0.63
	P_{neg}							**0.95**

[a] PABAK, P_{pos}, P_{neg} interpretation: <0.2 slight agreement, 0.2–0.4 = fair agreement, 0.4–0.6 = moderate agreement, 0.6–0.8 = substantial agreement, and >0.8 = almost perfect agreement (Dohoo et al., 2003).
[b] IFAT-A = IFAT, VMRD, Pullmann, WA, USA; ELISA-A1 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-B2 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-C = indirect ELISA, IDEXX Corp., Westbrook, ME, USA; IFAT-B = IFAT, USDA, Beltsville, MD, USA; NAT = *N. caninum* agglutination test, USDA, Beltsville, MD, USA.
[c] Cut-off value used: S/P ratio ≥0.6.

values. However, our main goal was to assess test performance and agreement of assays frequently used in research and diagnostic laboratories according to recommended cut-off values, not optimization of a commercially sold test. Appropriateness of a cut-off value depends on the purpose of performing the test, and must take into account the consequences of false-positive and false-negative outcomes. A previous study that also compared ELISA-B2 and ELISA-C (among others), found a higher kappa, Se and Sp compared to a competitive ELISA and immunoblotting analysis (Wu et al., 2002). However, a small reference sample set was used ($n = 30$), making estimates of test performance less precise. In addition, optimal cut-off values were determined for each test based on these reference sera, and were different from the cut-off value recommended by the manufacturers. Most variability in test performance was observed with respect to Se. In other studies (Dubey et al., 1997; Lally et al., 1996; Wouda et al., 1998), discrepancies in Se among tests were revealed especially when low titer sera were tested, highlighting the importance of selecting appropriate cut-off values for each test and each application. Concentration and type of antibodies present in the serum of chronically infected animals may differ compared to acute infections (Bjorkman et al., 1999), and therefore may be detected differently by certain antigens. With a serum dilution cut-off of 1:25 a moderate Se (0.66) for the NAT was observed. In a recent study (Canada et al., 2004) an optimal cut-off value of 1:40 was determined for the NAT. By using a lower dilution as a cut-off value, a higher Se and lower Sp was expected, but this was not observed (Table 2). The Youden's index was highest for ELISA-B2 and IFAT-B. The high index for IFAT-B indicates that an IFAT, when only taking test performance into account, unconstrained by factors such as cost and time, would be one of the preferred assays to use. However, the subjective evaluation of fluorescence in the IFAT-technique has to be considered. Experience of the person determining the degree of

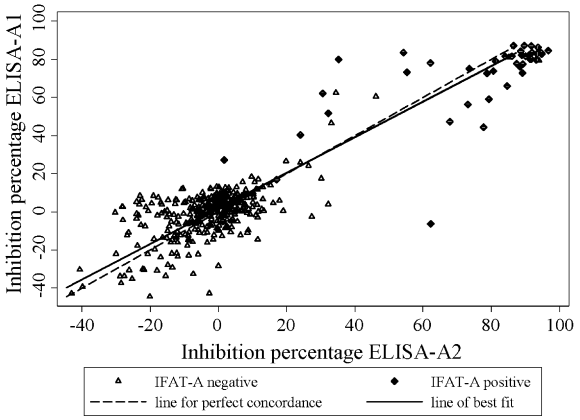

Fig. 2. Concordance correlation plot of a competitive *Neospora caninum* ELISA (ELISA-A1 and ELISA-A2) carried out at two different laboratories 2 years apart on sera from 397 dairy cows. IFAT-A = IFAT, VMRD Inc., Pullman, WA, USA; ELISA-A1 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-B2 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-C = indirect ELISA, IDEXX Corp., Westbrook, ME, USA.

fluorescence in the sample is critical, and reader fatigue does influence the outcome. Furthermore, IFATs are more time-consuming than ELISAs when analyzing large numbers of samples.

The NAT and the competitive ELISA have the advantage of not being host-species specific. As wildlife appears to be involved in the *N. caninum* epidemiology,

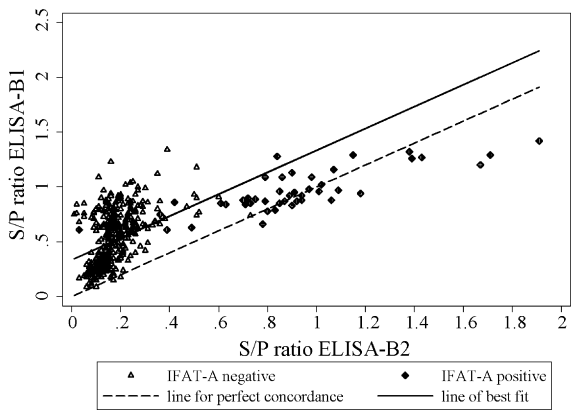

Fig. 3. Concordance correlation plot of an indirect *Neospora caninum* ELISA (ELISA-B1 and ELISA-B2) carried out at two different laboratories 6 years apart on sera from 397 dairy cows. IFAT-A = IFAT, VMRD Inc., Pullman, WA, USA; ELISA-A1 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-A2 = competitive inhibition ELISA, VMRD Inc., WA, USA; ELISA-B1 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-B2 = indirect ELISA, Biovet Inc., St. Hyacinthe, Que., Canada; ELISA-C = indirect ELISA, IDEXX Corp., Westbrook, ME, USA.

and considering there may be intermediate or definitive hosts not yet discovered, the option to test a variety of species with the same test can be advantageous. Assuming similar diagnostic performance of these two tests in other species, our results indicate that because of a higher Se, the competitive ELISA would be the preferred test to use.

Reproducibility of ELISA-A1 with ELISA-A2 was excellent (CCC = 0.9). The CCC of ELISA-B1 and ELISA-B2 was marginal (0.36), but this could be explained by an improvement of the laboratory protocol of this assay between the first and second analysis. A technical problem in the protocol of ELISA-B1 had been identified and corrected between the analysis of ELISA-B1 and ELISA-B2 (Dr. E. Cornaglia, personal communication). It is questionable whether the comparison of ELISA-B1 and ELISA-B2 is an appropriate reproducibility test, because improvements had been made to the test. This improvement was not known at the time of analysis, and the assay was commercially available under the same label and conditions in 1998 and 2004, and the test comparision was therefore considered a reproducibility test.

ELISA-B1 had a low Sp (0.52), combined with a Se of 1.00, thereby considerably overestimating the prevalence when used in a previous field-study using ELISA-B1(Keefe and VanLeeuwen, 2000). The manufacturer's Se and Sp estimates were 0.99 and 0.98, respectively (Biovet, 2006). An explanation for the high numbers of *N. caninum*-positive sera in the first assay performed in 1998 compared to other assays could be that antibody levels have diminished over the 6-year period of the study. However, a 2-year interval did not have any influence on sample analysis as shown by the high agreement between the first and second analysis of ELISA-A. Previous studies have shown that freezing does not have an appreciable influence on antibody levels (Wang et al., 1997), although frequent freeze–thaw cycles may decrease antibody titers in serum (Brey et al., 1994). Thus, it is likely that the manufacturer's Se and Sp estimates were inaccurate, and chances of misclassifying an uninfected animal as infected were considerable.

In conclusion the performance characteristics observed for most assays in this study, making them useful for screening antibodies to *N. caninum*.

Acknowledgements

We are grateful to the European *Neospora*-research group for providing the reference sera and to IDEXX Corp. and VMRD Inc. for their in-kind contribution. Drs. Javier Sanchez, Scott Adams, and Carey Wilson

are thanked for their technical assistance and kind participation in this study.

References

Baszler, T.V., Adams, S., Vander-Schalie, J., Mathison, B.A., Kostovic, M., 2001. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. J. Clin. Microbiol. 39, 3851–3857.

Baszler, T.V., Knowles, D.P., Dubey, J.P., Gay, J.M., Mathison, B.A., McElwain, T.F., 1996. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol. 34, 1423–1428.

Biovet Inc., Manual *N. caninum* antibody test kit (iELISA). Available at: http://www.biovet.ca/en/products/files/details.aspx?id=3&type=3 (accessed August 29, 2006)

Bjorkman, C., Naslund, K., Stenlund, S., Maley, S.W., Buxton, D., Uggla, A., 1999. An IgG avidity ELISA to discriminate between recent and chronic *Neospora caninum* infection. J. Vet. Diagn. Invest. 11, 41–44.

Brey, R.L., Cote, S.A., McGlasson, D.L., Triplett, D.A., Barna, L.K., 1994. Effects of repeated freeze–thaw cycles on anticardiolipin antibody immunoreactivity. Am. J. Clin. Pathol. 102, 586–588.

Byrt, T., Bishop, J., Carlin, J.B., 1993. Bias, prevalence and kappa. J. Clin. Epidemiol. 46, 423–429.

Canada, N., Meireles, C.S., Carvalheira, J., Rocha, A., Sousa, S., Correia da Costa, J.M., 2004. Determination of an optimized cut-off value for the *Neospora* agglutination test for serodiagnosis in cattle. Vet. Parasitol. 121, 225–231.

Cicchetti, D.V., Feinstein, A.R., 1990. High agreement but low kappa. II. Resolving the paradoxes. J. Clin. Epidemiol. 43, 551–558.

Dohoo, I.R., Martin, S.W., Stryhn, H., 2003. In: Dohoo, I.R. (Ed.), Veterinary Epidemiologic Research. AVC Inc., Charlottetown, p. 706.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Dubey, J.P., Jenkins, M.C., Adams, D.S., McAllister, M.M., Anderson-Sprecher, R., Baszler, T.V., Kwok, O.C., Lally, N.C., Bjorkman, C., Uggla, A., 1997. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. J. Parasitol. 83, 1063–1069.

Dubey, J.P., Lindsay, D.S., Adams, D.S., Gay, J.M., Baszler, T.V., Blagburn, B.L., Thulliez, P., 1996. Serologic responses of cattle and other animals infected with *Neospora caninum*. Am. J. Vet. Res. 57, 329–336.

Greiner, M., Pfeiffer, D., Smith, R.D., 2000. Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Prev. Vet. Med. 45, 23–41.

Keefe, G.P., VanLeeuwen, J.A., 2000. Neospora then and now: prevalence of *Neospora caninum* in Maritime Canada in 1979, 1989 and 1998. Can. Vet. J. 41, 864–866.

Lally, N.C., Jenkins, M.C., Dubey, J.P., 1996. Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. Clin. Diagn. Lab. Immunol. 3, 275–279.

Lin, L.I., 1989. A concordance correlation coefficient to evaluate reproducibility. Biometrics 45, 255–268.

Matsushita, S., Kashima, M., Joshima, H., 1987. Serodiagnosis of cilia-associated respiratory bacillus infection by the indirect immunofluorescence assay technique. Lab. Anim. 21, 356–359.

Pare, J., Hietala, S.K., Thurmond, M.C., 1995. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J. Vet. Diagn. Invest. 7, 352–359.

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.

VMRD Inc., Manual *N. caninum* antibody test kit (cELISA), Available at: http://vmrd.com/docs/tk/Neospora/Neospora_cELISA_2_and_5_plates_051027.pdf (accessed August 29, 2006).

Von Blumröder, D., Schares, G., Norton, R., Williams, D.J., Esteban-Redondo, I., Wright, S., Bjorkman, C., Frossling, J., Risco-Castillo, V., Fernandez-Garcia, A., Ortega-Mora, L.M., Sager, H., Hemphill, A., Van Maanen, C., Wouda, W., Conraths, F.J., 2004. Comparison and standardisation of serological methods for the diagnosis of *Neospora caninum* infection in bovines. Vet. Parasitol. 120, 11–22.

Waldner, C.L., Cunningham, G., Campbell, J.R., 2004. Agreement between three serological tests for *Neospora caninum* in beef cattle. J. Vet. Diagn. Invest. 16, 313–315.

Wang, G.R., Yang, J.Y., Lin, T.L., Chen, H.Y., Horng, C.B., 1997. Temperature effect on the sensitivity of ELISA, PA and WB to detect anti-HIV-1 antibody and infectivity of HIV-1. Zhonghua Yi. Xue. Za Zhi. (Taipei) 59, 325–333.

Wouda, W., Brinkhof, J., van Maanen, C., de Gee, A.L., Moen, A.R., 1998. Serodiagnosis of neosporosis in individual cows and dairy herds: a comparative study of three enzyme-linked immunosorbent assays. Clin. Diagn. Lab. Immunol. 5, 711–716.

Wouda, W., Dubey, J.P., Jenkins, M.C., 1997. Serological diagnosis of bovine fetal neosporosis. J. Parasitol. 83, 545–547.

Wu, J.T.Y., Dreger, S., Chow, E.Y.W., Bowlby, E.E., 2002. Validation of 2 commercial *Neospora caninum* antibody enzyme linked immunosorbent assays. Can. J. Vet. Res. 66, 264–271.

Youden, W.J., 1950. Index for rating diagnostic tests. Cancer 3, 32–35.